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THE ROLE OF RECOMBINANT Rb GENE ADENOVIRUS VECTOR IN THE GROWTH OF LUNG ADENOCARCINOMA CELLS*

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Objective: To study the role of the most extensively studied tumor suppressor gene, retinoblastoma (Rb) gene, on the growth of lung adenocarcinoma cell line GLC-82 and explore a gene therapy approach for lung adenocarcinoma. Methods: The recombinant Rb gene adenovirus vector was constructed, the control virus which carries LacZ gene was producted by the same method. Infection effects were detected by biochemical staining of β-gal and immunohistochemical analysis of Rb protein. The Rb cDNA of infected cells were determined by PCR. The cell growth rate and cell cycle were observed by cell-counting and flow cytometry. Results: The constructed recombinant adenovirus vector could infect effectively the cells with high level expression of Rb cDNA and Rb protein. The transfection of wild-type Rb gene could suppress GLC-82 cell proliferation and decrease the cellular DNA synthesis. Conclusions: These results showed the possibility of using recombinant Rb gene adenovirus vector in the gene therapy of cancer to inhibit the growth of cancer.

Key words: Adenovirus vector, Retinoblastoma gene, GLC-82 cell

The retinoblastoma (Rb) gene encoding a protein of 928 amino acids can inhibit tumor formation. The deficient and inactivation of Rb gene is related to the initiation and progression of many common cancer.[1-5] In some laboratories in China, vector expressing Rb gene was constructed and mutation of Rb gene was observed in tumor cells from widely disparate types of numan cancers.[6,7] In the present study, we constructed a replication-deficient recombinant adenovirus vector encoding a wild-type Rb protein and investigated the efficiency of Rb gene transfection and the inhibition on growth of lung adenocarcinoma cell line GLC-82, and explored a gene therapy approach for lung cancer.

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MATERIALS AND METHODS

Construction of Recombinant Rb Adenovirus Vector

A replication-deficient adenovirus vector encoding a wild-type Rb protein, which contains the human cytomegalovirus (CMV) promotor, the Rb cDNA fragment and the polyadenylation site of the rabbit β-globin gene, was constructed as previously described[8] and designated Ad Δ Rb. High-titer stock of Ad Δ Rb virus contained 10^{12} viral particles/ml as measured by absorbance at 260nm (1 A260 $=1\times10^{12}$ viral particles/ml). The replication-deficient recombinant adenovirus containing the LacZ gene (Ad Δ LacZ), which was used as a control in the experiments,

was generated by the same methods.

Culture of Lung Adenocarcinoma Cell Line GLC-82

Human lung adenocarcinoma cell line GLC-82 was generously provided by Cancer Hospital, Chinese Academy of Medical Sciences, and was maintained in RPMI 1640 medium containing 10% fetal bovine serum.

Determination of Exogenous Rb cDNA

Genomic DNA was prepared from infected and noninfected GLC-82 cells. Oligonucleotide primers were synthesized, corresponding to human Rb nucleotides 1168-1187 and nucleotides 1480-1462. A PCR was performed with 100 ng of genomic DNA for 30 cycles of denaturation (95°C, 1 min), annealing (50°C, 30 sec), and polymerization (72°C, 1min). PCR products were analyzed by ethidium bromide staining on a 1% agarose gel.

Examination of Endogenous Rb Gene

Genomic DNA prepared from GLC-82 cells was analyzed by PCR with oligonucleotide primers corresponding to human Rb nucleotides in exon 8.

Observation for Gene Expression Efficiency of Recombinant Adenovirus

The GLC-82 (1×10^5 cells/well) were seeded on coverslips in six-well culture plates and cultured for 24h. After infection for 72h with Ad Δ LacZ or Ad Δ Rb, the cells were fixed with aceton and formaldenyde. The expression of β-gal in Ad Δ LacZ virus-transduced cells was detected by staining the cells with x-gal for 4h at 37°C. The efficiency of gene transfection was quantitated by counting the percentage of blue cells. Immunochemical staining for detection of Rb protein in cells was performed using the anti-Rb monoclonal antibody (Triton Biosciences).

Growth Curve of GLC-82 Cells

GLC-82 cells (0.6×10^5 cells/well) were plated in 24-well plates and allowed to adhere 24h at 37°C. The cells were then infected with Ad Δ Rb or Ad Δ LacZ for an additional 24, 48, 72, 96 and 120h of incubation at 37°C. The cell numbers were counted each day for 5 days.

Flow cytometry Analysis

Single cell suspensions collected at each time point were fixed with 70% ethanol. After incubation in 200 μg/ml RNase for 30 min at 37°C, the cells were suspended in 50 μg/ml propidium iodide for 30 min at 4°C and then subjected to flow cytometry.

RESULTS

Efficiency of Recombinant Adenovirus Infection

To determine the efficiency of adenovirus infection, cultured GLC-82 cells were infected for 24h with Ad Δ Rb or Ad Δ LacZ at different concentrations from 0 to 10^8 pfu/ml. The results showed that infection of GLC-82 with Ad Δ LacZ at the concentration of 10^7 pfu/ml leads to the transduction of 100% of GLC-82. Rb gene expression efficiency in GLC-82 cells infected with Ad Δ Rb at the concentration of 10^8 pfu/ml was more than 80%.

Analysis of Rb cDNA

Successful gene transfer into GLC-82 cells was analyzed by PCR using oligometric primers specific for human Rb cDNA. As shown by agarose gel electrophoresis (Figure 1), Rb cDNA presents in GLC-82 cells infected with Ad Δ Rb. A 327 bp product of PCR was not obtained in uninfected GLC-82.

Detection of Rb Gene and Rb Protein in GLC-82

The result of PCR for Rb gene in GLC-82 cells showed that a product of PCR was obtained in uninfected GLC-82, suggesting that endogenous Rb gene presents in cultured GLC-82 cells. The expression of Rb in Ad Δ Rb-infected and uninfected GLC-82 was comfirmed by immunochemical staining. As expected, Ad Δ Rb-infected GLC-82 displayed high levels of expression of Rb protein, while Rb expression was not found in uninfected GLC-82.

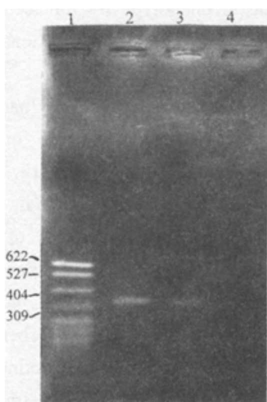

Fig 1. Detection of PCR products of Rb cDNA by 1% agarose gel electrophoresis

Lane 1 : PBR 322/MspI marker;
Lane 2: Positive control (pRC/CMV Rb);
Lane 3: PCR products of Ad Δ Rb-infected cells;
Lane 4: PCR products of uninfected cells.

Effect of Rb Expression on Cell Growth

To further test the activity of Rb recombinant adenoviruses, we assayed their ability to inhibit proliferation of GLC-82. As shown in Figure 2, the uninfected and Ad Δ LacZ-infected cells proliferated rapidly during the first 96h. In contrast, infection with Ad Δ Rb resulted in a 70.6% reduction in GLC-82 growth at 96h.

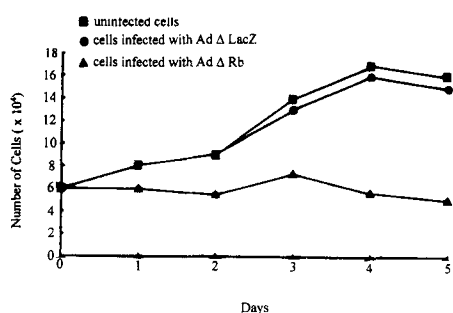

Fig 2. Effect of Rb gene on the growth of GLC-82 cells

-■- uninfected cells
-●- cells infected with Ad Δ LacZ
-▲- cells infected with Ad Δ Rb

Flow Cytometric Determination of Cell Cycle Progression

As shown in Figure 3, 72h after infection with Ad Δ Rb, the number of cells in Go/G1 phase of the cell cycle increased. In contrast, the number of cells in G2/M and S phase reduced. These results revealed that introduction of Rb gene via a recombinant adenovirus vector suppresses growth of GLC-82 cells through regulation of DNA synthesis and cell cycle progression.

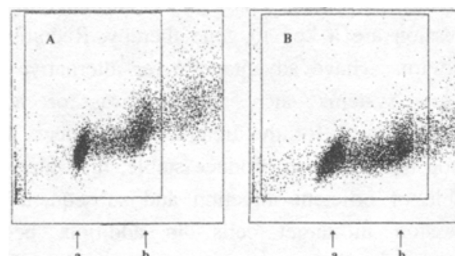

Fig 3. Analysis of cell cycle progression by flow cytometry

A: uninfected GLC-82; B: Ad Δ Rb-infected GLC-82;
a : cells in G0/G1 phase; b: cells in G2/M phase

DISCUSSION

Inactivation of the tumor suppressor genes involved in tumorigenesis. Replacement of suppressor genes in tumor cells could be a novel strategy for the treatment of clinical malignancy.[1] Unlike conventional, cytotoxic cancer therapies, gene therapy would be based on permanent correction of an underlying defect in tumor cells. Therapy may not need to be targeted because cancer suppressor genes should not harm normal cells. The ultimate utility of this approach will depend on progress in obtaining other cancer suppressor genes, in understanding their involvement in human tumors, and in finding the safe and effective technology of exogenous gene transfection and expression.

The deficient and inactivation of Rb gene have been found in different types of tumor cells. A protein product of Rb gene, a nuclearphosphoprotein of 928 amino acids, can form complexes with specific cellular transcription factor and bind to DNA. Thus, Rb protein inhibits DNA synthesis and arrests the cells in G0/G1 phase of the cell cycle.[9] In this study, we noted that endogenous Rb gene presents in cultured GLC-82 cells, while no Rb protein was detected in GLC-82 by

immunochemical staining. It is likely due to low level of Rb expression or short half-life of Rb protein. The introduction and expression of exogenous Rb gene can suppress GLC-82 growth. Therefore, we suggested that tumor cells were remained in G0/G1 phase and cell proliferation was inhibited by introduction and expression of exogenous Rb gene, no matter whether endogenous Rb gene presents in cells.

An appropriate vector and approach for high efficiency of exogenous gene introduction and expression are a key to gene therapy. Recombinant adenoviruses have advantages over alternative gene delivery systems such as retrovirus or adeno-associated virus for the treatment of cancer. These include the ability to produce stable, high-titer virus capable of efficient infection and subsequent gene expression in target cells. In addition, because adenoviruses do not integrate into the cellular chromosome, the chance of insertional mutagenesis is minimized and permanent expression of their DNA does not occur. These features make adenoviral vectors suitable gene delivery system for the potential gene therapy of cancer. Thus, we constructed recombinant adenovirus encoding wild-type Rb which can transfect effectively into the GLC-82 with high level expression of Rb protein. The results indicated that introduction of Rb gene mediated by adenovirus could be a gene therapy approach for cancer.

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